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Lipid peroxidation and haemoglobin degradation in red blood cells exposed to t-butyl hydroperoxide

Effects of the hexose monophosphate shunt as mediated by glutathione and ascorbate

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Lipid peroxidation and haemoglobin degradation were the two extremes of a spectrum of oxidative damage in red cells exposed to t-butyl hydroperoxide. The exact position in this spectrum depended on the availability of glucose and the ligand state of haemoglobin. In red cells containing oxy- or carbonmono-oxy-haemoglobin, hexose monophosphate-shunt activity was mainly responsible for metabolism of t-butyl hydroperoxide; haem groups were the main scavengers in red cells containing methaemoglobin. Glutathione, via glutathione peroxidase, accounted for nearly all of the hydroperoxide metabolizing activity of the hexose monophosphate shunt. Glucose protection against lipid peroxidation was almost entirely mediated by glutathione, whereas glucose protection of haemoglobin was only partly mediated by glutathione. Physiological concentrations of intracellular or extracellular ascorbate had no effect on consumption of t-butyl hydroperoxide or oxidation of haemoglobin. Ascorbate was mainly involved in scavenging chain-propagating species involved in lipid peroxidation. The protective effect of intracellular ascorbate against lipid peroxidation was about 100% glucose-dependent and about 50% glutathione-dependent. Extracellular ascorbate functioned largely without a requirement for glucose metabolism, although some synergistic effects between extracellular ascorbate and glutathione were observed. Lipid peroxidation was not dependent on the rate or completion of t-butyl hydroperoxide consumption but rather on the route of consumption. Lipid peroxidation appears to depend on the balance between the presence of initiators of lipid peroxidation (oxyhaemoglobin and low concentrations of methaemoglobin) and terminators of lipid peroxidation (glutathione, ascorbate, high concentrations of methaemoglobin).

Lipid peroxidation has been observed *in vivo* in pathological states (see Plaa & Witschi, 1976) and as a physiologically ongoing process (see Tappel, 1980). Lipid peroxidation has many deleterious effects on membrane structure and function (Vladimirov *et al.*, 1980; Menzel, 1980). Peroxidizing lipids generate many potentially cytotoxic products (see Logani & Davies, 1980; Benedetti *et al.*, 1979); stoichiometrically the major products are lipid hydroperoxides. Lipid hydroperoxides are highly toxic *in vivo* (Horgan *et al.*, 1957) and *in vitro* are capable of inactivating enzymes (Chio & Tappel, 1969; Green *et al.*, 1971), covalently altering biomolecules (Lewis & Wills, 1962; Nielson, 1981) and initiating free-radical mediated destruction of proteins (see Logani & Davies, 1980) and unsaturated lipids (Shimasaki & Privett, 1975).

We have studied red cells exposed to t-butyl hydroperoxide as a model for the effects of lipid peroxidation in a metabolically active cell. Externally added t-butyl hydroperoxide may qualify as a model for endogenous lipid hydroperoxides (Sies & Summer, 1975). The red cell is normally exposed to high concentrations of O_2 and has a membrane rich in polyunsaturated lipids in close apposition to a concentrated solution of haem-protein, a powerful catalyst of lipid peroxidation (Wills, 1965). We have previously shown that exposure of red cells con-

Abbreviations used: DETAPAC, diethylenetriamine penta-acetic acid; HbO_2, oxyhaemoglobin; metHb, methaemoglobin; HbCO, carbonmono-oxyhaemoglobin; intact Hb, intact haemoglobin (defined as the sum of HbO_2 plus metHb); TBAR, thiobarbituric acid-reactive products of lipid peroxidation; NEM, *N*-ethylmaleimide; GSSG, oxidized glutathione; GSH, reduced glutathione; t-Bu, t-butyl.

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taining HbO_2, metHb or HbCO to t-butyl hydroperoxide resulted in lipid peroxidation and haemoglobin degradation as the two extremes of a spectrum of oxidative damage (Trotta *et al.*, 1981). The exact position in this spectrum depended on the availability of glucose and the ligand state of haemoglobin.

In the present study we have examined the role of the hexose monophosphate shunt in the metabolism of t-butyl hydroperoxide and protection against lipid peroxidation and haemoglobin alterations. The requirement for glutathione as a mediator of the effects of the hexose monophosphate shunt was studied using red cells pretreated with NEM to inactivate glutathione. Glutathione has been shown to be of importance during oxidative stress because it supplies glutathione peroxidase (which utilizes H_2O_2 and fatty acid hydroperoxides as substrates) with reducing equivalents (Christopherson, 1969), maintains protein sulphydryl groups in reduced form (Flohé & Günzler, 1976) and may non-enzymically inactivate free radicals (Kosower, 1976). The role of ascorbate was studied by using red cells pre-incubated with ascorbate to enrich the intracellular ascorbate pool or by carrying out incubations of red cells with t-butyl hydroperoxide in a medium containing a physiological (plasma) concentration of ascorbate. It has been postulated that ascorbate serves as an important antioxidant defence because it has been shown to scavenge superoxide radicals (Nishikimi, 1975) and is an excellent free-radical quencher (Redpath & Willson, 1973). In addition, it is postulated that ascorbate reduces tocopheryl radical to tocopherol and augments the role of the latter as an inhibitor of lipid peroxidation (Packer *et al.*, 1979).

Materials and methods

Red cell preparations

Adult human blood was drawn daily into a test tube containing a small amount of 3.8% sodium citrate solution. After centrifugation, plasma and white cells were removed and red cells were washed three times with phosphate-buffered saline (nine parts 0.9% NaCl, one part 0.1 M-KH_2PO_4/K_2HPO_4, pH 7.4). Experiments were carried out with three types of red-cell preparations, characterized by the haemoglobin type (HbO_2, metHb or HbCO). Red cells containing HbO_2 were taken directly from the washed red-cell pellets. Red cells containing metHb were prepared by incubating a volume of packed red cells with an equal volume of 0.5% $NaNO_2$ in half-concentrated phosphate-buffered saline for 10 min at 25°C. After incubation, red cells were washed five times with phosphate-buffered saline to remove nitrite. Stock red-cell suspensions containing HbCO were prepared by blowing CO over a 20% (v/v) suspension of red cells in phosphate-buffered saline until the visible spectra of red-cell lysates reached a maximum at 569 nm. Incubations of red cells containing HbCO were carried out in air-tight vials under an atmosphere of 90% air plus 10% CO.

Preparation of ascorbate-enriched red cells and inactivation of intracellular glutathione by treatment with NEM

Ascorbate-enriched red cells were prepared from red cells containing either HbO_2 or metHb by pre-incubation with ascorbate. A 10% (v/v) red cell suspension (10 ml) in Krebs–Ringer phosphate buffer (120 mM-NaCl, 4.8 mM-KCl, 1.3 mM-$CaCl_2$, 1.2 mM-$MgSO_4$, 16.5 mM-NaH_2PO_4/Na_2HPO_4, pH 7.4) containing 5 mM-glucose was mixed with 0.1 ml of 50 mM-sodium ascorbate (Sigma, St. Louis, MO, U.S.A.) and incubated for 45 min at 37°C in a shaking water bath. Control (incubated without ascorbate) and ascorbate-enriched red cells were then centrifuged and the 1 ml packed red-cell pellet was resuspended in Krebs–Ringer phosphate buffer containing 5 mM-glucose to give 5 ml of 20% (v/v) red-cell suspension. Two portions, each containing 2 ml of 20% (v/v) red-cell suspension, from both control and ascorbate-enriched red cells were transferred to 10 ml Erlenmeyer flasks. One of the portions was mixed with a stock solution of NEM (Sigma). The NEM stock solution, 20 mM-NEM in phosphate-buffered saline (pH 5.3), was prepared just before addition. Enough NEM was added to the red-cell suspension to bring the final concentration to 1.42 μmol of NEM/μmol of glutathione [based on glutathione assays as described by Beutler (1975)]. NEM at 1.35 μmol/μmol of glutathione was required to bind all the intracellular glutathione; 5% additional NEM (0.07 μmol) was added to ensure completion of the reaction. All four portions of red-cell suspensions were incubated for 10 min at 37°C in a shaking water bath. For experiments requiring red cells containing HbCO, incubation of red cells containing HbO_2 was carried out under an atmosphere of 100% CO. After incubation, red-cell suspensions were centrifuged, red cells were washed once with phosphate-buffered saline and resuspended in phosphate-buffered saline to give 20% (v/v) red-cell suspensions. These procedures yielded four types of red-cell preparations: control, ascorbate-enriched, NEM-treated and ascorbate-enriched plus NEM-treated. Intracellular total ascorbate (defined as reduced ascorbate plus dehydroascorbate plus dioxogulonate) was assayed in each of these preparations by the method of Chatterjee & Banerjee (1979).

Incubations with t-butyl hydroperoxide

Incubations were carried out in 25 ml flasks

containing 0.1 ml of 2 mM-t-butyl hydroperoxide (Sigma) in phosphate-buffered saline and 0.1 ml of 20% (v/v) red-cell suspension in phosphate-buffered saline brought to a final volume of 2 ml with Krebs–Ringer phosphate buffer. The final concentrations were 0.1 mM-t-butyl hydroperoxide and 1% (v/v) red cells. Variable additions included (at final concentrations) 3.75 mM-glucose and 0.025 mM-ascorbate. Stock ascorbate solutions (1 mM) were prepared in phosphate-buffered saline (pH 2.3). After completing all additions, the 25 ml reaction flasks were closed with rubber stoppers, and in the case of red cells containing HbCO, 2.5 ml of CO was injected to give a final gas-phase composition of 9 : 1 (air/CO). Flasks were incubated for 1 h at 37°C in a shaking water bath. At 1 h the incubation mixture was centrifuged at 1200 *g* for 5 min. Supernatants were removed and used for measurement of residual t-butyl hydroperoxide and thiobarbituric acid-reactive products of lipid peroxidation. Red-cell pellets were used for studies of haemoglobin.

Measurement of lipid peroxidation

TBAR products were assayed by a modification of the technique of Stocks & Dormandy (1971). The 2 ml supernatant fraction was mixed with 1 ml of 30% trichloroacetic acid and centrifuged at 5000 *g* for 15 min. A portion (2 ml) of this supernatant and 0.5 ml of 1% thiobarbituric acid (Baker, Phillipsburg, NJ, U.S.A.) in 0.05 M-NaOH were added to a loosely stoppered test tube and heated in a boiling-water bath for 10 min. The test tubes were cooled under tap water and absorption spectra were recorded between 500 and 600 nm in a Cary 14 spectrophotometer using a cuvette with a 1 cm light-path. The pink chromophore was stable for 1 h at 25°C. The difference in A_{532} between samples and respective controls without t-butyl hydroperoxide was used to calculate nM concentrations of TBAR products. For these calculations, standard curves were prepared by using malonaldehyde prepared from 1,1,3,3-tetramethoxypropane (Aldrich, Milwaukee, WI, U.S.A.) (Placer *et al.*, 1966). Lack of inhibition of supernatant assays by butylated hydroxytoluene (Aldrich) or DETAPAC indicated that TBAR products were largely free malonaldehyde and not its precursors (Asakawa & Matsushita, 1980; Gutteridge, 1981).

Analysis of red-cell haemoglobin

Red-cell haemoglobin was analysed by a modification of the procedure of Harley & Mauer (1960). The 0.02 ml packed red-cell fraction was washed with phosphate-buffered saline followed by lysis with 4 ml of water and the addition of 2 ml of 60 mM-NaH_2PO_4/Na_2HPO_4, pH 7.4. Lysates were centrifuged to remove membranous material. In the case of lysates prepared from red cells containing HbCO, lysates were further incubated at 25°C with gentle shaking for 1 h under air until the visible spectra reached a maximum at 577 nm showing restoration of HbO_2. HbO_2 concentration was measured by observing the increase in absorbance at 620 nm of red-cell lysates after the addition of $Fe(CN)_6^{3-}$. MetHb concentration was measured by observing the decrease in absorbance at 620 nm after the addition of CN^-. Intact Hb concentration was measured directly by observing the decrease in absorbance at 620 nm after the addition of CN^- to lysates treated previously with $Fe(CN)_6^{3-}$. The results of direct measurement of intact Hb were checked with the calculated sum, HbO_2 plus metHb. A decrease in intact Hb concentration during incubation of red cells was taken as evidence for accumulation of haemoglobin metabolites (other than HbO_2 and metHb), designated below as non-intact Hb.

Assay for t-butyl hydroperoxide

Portions (1 ml) of supernatants from incubations of red cells with t-butyl hydroperoxide were mixed with 0.1 ml of 50% trichloroacetic acid and assayed for residual t-butyl hydroperoxide by a modification of the technique of Hicks & Gebicki (1979). A 3 ml cuvette was prepared containing a stirring bar and 1.8 ml of N_2-sparged methanol/acetic acid (1 : 1, v/v) with N_2 gas continuously bubbled through the solution. A solution (1.9 ml) saturated with both KI (Sigma) and EDTA in N_2-sparged methanol was added and the cuvette was stoppered, being careful to exclude air. The contents were mixed and the change in absorbance at 360 nm was monitored in a Cary 14 spectrophotometer. A linear rate of absorbance increase of about 0.005 A_{360}/10 min was generally observed due to traces of O_2. Samples containing t-butyl hydroperoxide were vigorously mixed with N_2 for 1 min and 0.1 ml portions were injected into the cuvette. The increase in A_{360} was followed for 10 min. The background absorbance change was subtracted to obtain the absorbance change due to t-butyl hydroperoxide. A linear standard curve was obtained using known concentrations of t-butyl hydroperoxide. The limit of detection was about 5 μM-t-butyl hydroperoxide in the injected 0.1 ml sample portion.

Measurement of flux of glucose through the hexose monophosphate shunt

The flux of glucose through the hexose monophosphate shunt was measured by collecting $^{14}CO_2$ released from D-[U-^{14}C]glucose. (U-^{14}C)-labelled glucose was used instead of (1-^{14}C)-labelled glucose so that the entire flux was measured, including recycling of pentoses through the hexose monophosphate shunt. Conditions were identical with other red-cell incubations except that the final

concentration of 3.75 mM-D-glucose included 0.33 μCi of D-[U-^{14}C]glucose (New England Nuclear, Boston, MA, U.S.A.). Blanks were prepared by adding 1 ml of 10% $HClO_4$ to red-cell suspensions before the addition of D-[U-^{14}C]glucose. Flasks were stoppered with wells containing 0.2 ml of 2 M-KOH and incubated for 1 h at 37°C in a shaking water bath. The reaction was stopped by injection of 1 ml of 10% $HClO_4$ and the incubation was continued for 30 min to ensure that released $^{14}CO_2$ was trapped in the KOH. Contents of the well (KOH with trapped $^{14}CO_2$) were transferred into 5 ml of Oxosol (National Diagnostic, Somerville, NJ, U.S.A.) and counted for radioactivity in a liquid-scintillation counter. The flux through the hexose monophosphate shunt was calculated after subtracting blank values and expressed as μmol of CO_2 produced/h per ml of red cells.

Incubations of t-butyl hydroperoxide with ascorbate in the absence of red cells

Incubations of t-butyl hydroperoxide with ascorbate in the absence of red cells were otherwise identical with incubations in the presence of red cells. Assays for total ascorbate and t-butyl hydroperoxide were as described above except that for measurements of t-butyl hydroperoxide excess catalase (88 823 units/mg; Calbiochem, La Jolla, CA, U.S.A.) was added to remove H_2O_2. The loss of reduced ascorbate was continuously monitored by observing the change in absorbance at 266 nm in a Cary 14 spectrophotometer.

Statistical analyses

The significance of differences between observations on red-cell incubations under different conditions was determined by using a paired *t* test. The resulting *P* values are indicated in parentheses in the Results section together with the number of paired experiments.

Results

Ascorbate content of red-cell preparations

Ascorbate content of red-cell preparations was altered by treatment with NO_2^- to make metHb-containing red cells, with ascorbate to increase the intracellular ascorbate pool and with NEM to inactivate glutathione. Table 1 shows the final ascorbate concentrations in these various red-cell preparations. Treatment of HbO_2-containing red cells with NO_2^- to make metHb-containing red cells resulted in a 50% loss of ascorbate. Incubation of red cells in a medium containing ascorbate enriched intracellular ascorbate 2-fold in HbO_2-containing red cells and 10-fold in metHb-containing red cells. Treatment with NEM had no effect on ascorbate in control red cells containing either HbO_2 or metHb, but significantly decreased ascorbate in ascorbate-enriched red cells. Although treatment with NEM resulted in loss of ascorbate in ascorbate-enriched red cells containing either HbO_2 or metHb, these red cells remained enriched with ascorbate relative to controls. The ascorbate content of HbCO-containing red cells was similar to that of HbO_2-containing red cells, since the transition from HbO_2 to HbCO was carried out by incubation under CO after incubation with ascorbate or during incubation with NEM.

Table 1. *Ascorbate content of red cell preparations*

Red cells containing HbO_2 were incubated with NO_2^- to make metHb-containing red cells, with ascorbate to increase the intracellular ascorbate pool and with NEM to inactivate glutathione. The various red cell preparations were assayed for total ascorbate (defined as reduced ascorbate plus dehydroascorbate plus dioxogulonate) as described in the Materials and methods section.

		Total ascorbate (μmol/ml of red cells)	
Red cell haemoglobin	Red cell preparation	Expt. 1	Expt. 2
HbO_2 (HbCO)*	Control	0.051	0.086
	Ascorbate-enriched	0.113	0.167
	NEM-treated	0.046	0.090
	Ascorbate-enriched plus NEM-treated	0.098	0.149
MetHb	Control	0.024	0.045
	Ascorbate-enriched	0.324	0.379
	NEM-treated	0.022	0.052
	Ascorbate-enriched plus NEM-treated	0.120	0.181

* The ascorbate content of HbCO-containing red cells was similar to that of HbO_2-containing red cells since the transition from HbO_2 to HbCO was carried out by incubation under CO after incubation with ascorbate or during incubation with NEM.

Effects of t-butyl hydroperoxide on HbO_2-containing red cells

Table 2 reports lipid peroxidation, haemoglobin changes, and flux through the hexose monophosphate shunt in HbO_2-containing red cells. As previously described (Trotta *et al.*, 1981), t-butyl hydroperoxide-induced lipid peroxidation in HbO_2-containing red cells was increased by the presence of glucose, whereas formation of metHb and non-intact Hb was decreased. Treatment with NEM to remove glutathione completely abolished the protection of haemoglobin by glucose. Ascorbate-enriched red cells in the presence of glucose were partially protected against lipid peroxidation relative to controls ($P < 0.05$; four paired experiments). Ascorbate-enriched red cells lacked protection against lipid peroxidation when glucose was absent and exhibited only slight protection when glutathione was removed by treatment with NEM. When a physiological concentration of ascorbate was included in the medium, lipid peroxidation was greatly inhibited, whether or not glucose was present or red cells were treated with NEM. Neither ascorbate-enrichment nor extracellular ascorbate had any effect on haemoglobin changes.

In HbO_2-containing red cells, t-butyl hydroperoxide caused a 7-fold increase in flux through the hexose monophosphate shunt. Stimulation of hexose monophosphate shunt activity was decreased slightly in ascorbate-enriched red cells and decreased by 40% by extracellular ascorbate. Treatment with NEM completely abolished t-butyl hydroperoxide-stimulation of the hexose monophosphate shunt. By 1 h at 37°C, nearly all of the t-butyl hydroperoxide had been consumed by HbO_2-containing red cells (less than 5% remaining) regardless of the red-cell preparation or additions to the incubation medium (data not shown).

Effects of t-butyl hydroperoxide on metHb-containing red cells

The effects of t-butyl hydroperoxide on metHb-containing red cells are shown in Table 3. As previously shown (Trotta *et al.*, 1981), metHb-containing red cells are resistant to lipid peroxidation relative to HbO_2-containing red cells. The presence of glucose had little effect on either lipid peroxidation or haemoglobin changes in metHb-containing red cells. Glucose caused a small but significant inhibition of formation of non-intact Hb ($P < 0.001$; six paired experiments), which was

Table 2. *Effects of t-butyl hydroperoxide on HbO_2-containing red cells*

A 1% (v/v) suspension of red cells containing HbO_2 was exposed to 0.1 mM-t-butyl hydroperoxide for 1 h at 37°C. Before incubation some red cell preparations were pre-incubated with ascorbate to increase the intracellular ascorbate pool (ascorbate-enriched) and/or pre-incubated with NEM to inactivate glutathione. Where indicated, 3.75 mM-glucose and/or 0.025 mM-ascorbate were present in the medium during exposure to t-butyl hydroperoxide. After exposure to t-butyl hydroperoxide, measurements were made of lipid peroxidation (estimated by assay of the TBAR), levels of metHb and non-intact Hb and flux of glucose through the hexose monophosphate shunt (HMS).

Red cell preparation	Additions to incubation medium	TBAR products (nM)* Set 1	TBAR products (nM)* Set 2	Haemoglobin† MetHb (%) Set 1	Haemoglobin† MetHb (%) Set 2	Haemoglobin† Non-intact Hb (%) Set 1	Haemoglobin† Non-intact Hb (%) Set 2	Flux through the HMS (μmol of CO_2/h per ml of red cells)‡
Control	None	325 ± 24	378 ± 30	56.0	59.1	13.2	29.8	
Control	Ascorbate		50 ± 12		59.2		24.3	
Ascorbate-enriched	None	340 ± 27		56.9		15.0		
NEM-treated	None	367 ± 38	417 ± 3	59.4	58.8	16.9	29.6	
NEM-treated	Ascorbate		49 ± 33		58.5		29.8	
Ascorbate-enriched/ NEM-treated	None	409 ± 56		63.0		17.1		
Control	Glucose	339 ± 46	440 ± 73	28.4	37.6	5.2	16.9	3.00
Control	Glucose + ascorbate		57 ± 17		37.7		14.4	1.84
Ascorbate-enriched	Glucose	265 ± 51		30.4		5.2		2.47
NEM-treated	Glucose	436 ± 70	485 ± 27	52.8	56.6	16.3	31.4	0.46
NEM-treated	Glucose + ascorbate		171 ± 43		56.8		27.1	0.20
Ascorbate enriched/ NEM-treated	Glucose	384 ± 25		62.7		12.4		0.40

* Data are means of four experiments (set 1) or three experiments (set 2) ± S.E.M.

† Data are means of two experiments.

‡ Data are means of two experiments. Flux through the HMS in the absence of t-butyl hydroperoxide was 0.42 ± 0.09 (mean ± S.E.M., $n = 6$) in control preparations and 0.38 ± 0.10 ($n = 6$) in NEM-treated preparations.

Table 3. *Effects of t-butyl hydroperoxide on metHb-containing red cells*

A 1% (v/v) suspension of red cells containing metHb was exposed to 0.1 mM-t-butyl hydroperoxide for 1 h at 37°C. Conditions and procedures are described in the legend to Table 2.

Red cell preparation	Additions to incubation medium	TBAR products (nM)* Set 1	TBAR products (nM)* Set 2	Haemoglobin† MetHb (%) Set 1	MetHb (%) Set 2	Non-intact Hb (%) Set 1	Non-intact Hb (%) Set 2	Flux through the HMS (μmol of CO_2/h per ml of red cells)‡	Residual t-BuOOH (mM)§
Control	None	190 ± 25	130	59.1	56.7	38.6	42.9		<0.005
Control	Ascorbate		80		59.2		40.8		<0.005
Ascorbate-enriched	None	215 ± 44		62.1		34.7			<0.005
NEM-treated	None	206 ± 32	182	54.2	52.7	43.9	47.4		0.014
NEM-treated	Ascorbate		90		55.7		42.2		0.008
Ascorbate-enriched/ NEM-treated	None	199 ± 30		53.0		45.8			0.014
Control	Glucose	214 ± 12	176	57.2	58.2	33.1	38.6	0.77	<0.005
Control	Glucose + ascorbate		64		59.0		35.5	0.81	<0.005
Ascorbate-enriched	Glucose	159 ± 26		56.1		32.2		0.75	<0.005
NEM-treated	Glucose	191 ± 9	132	54.0	51.0	40.5	46.4	0.48	<0.005
NEM-treated	Glucose + ascorbate		124		57.0		42.3	0.44	<0.005
Ascorbate-enriched/ NEM-treated	Glucose	168 ± 9		54.3		39.6		0.44	<0.005

* Data are means of four experiments ± S.E.M. (set 1) or two experiments (set 2).

† Data are means of three experiments (set 1) or two experiments (set 2).

‡ Data are means of two experiments. Flux through the HMS in the absence of t-butyl hydroperoxide was 0.50 ± 0.14 (mean ± S.E.M., $n = 6$) in control preparations and 0.28 ± 0.09 ($n = 6$) in NEM-treated preparations.

§ Data are means of two experiments.

abolished by treatment with NEM. The effects of ascorbate-enrichment or extracellular ascorbate on lipid peroxidation in metHb-containing red cells were qualitatively similar to the effects observed in HbO_2-containing red cells. Ascorbate-enriched red cells were partially protected against lipid peroxidation in the presence of glucose ($P < 0.05$; four paired experiments). Ascorbate-enriched red cells lacked protection against lipid peroxidation when glucose was absent and exhibited only slight protection when glutathione was removed by treatment with NEM. Extracellular ascorbate inhibited lipid peroxidation whether or not glucose was present or red cells were treated with NEM. In metHb-containing red cells (as in HbO_2-containing red cells) neither ascorbate-enrichment nor extracellular ascorbate had any effect on haemoglobin changes.

In metHb-containing red cells, t-butyl hydroperoxide caused only a modest increase (50%) in flux through the hexose monophosphate shunt compared with HbO_2-containing red cells (600%). This 50% stimulation persisted in red cells treated with NEM and was unaffected by ascorbate-enrichment or extracellular ascorbate. By 1 h at 37°C, nearly all of the t-butyl hydroperoxide had been consumed by metHb-containing red cells with the exception of incubation mixtures of NEM-treated red cells in the absence of glucose, which contained 14% of the original t-butyl hydroperoxide at 1 h.

Effects of t-butyl hydroperoxide on HbCO-containing red cells

The effects of t-butyl hydroperoxide on HbCO-containing red cells are shown in Table 4. We have previously shown that HbCO-containing red cells sustain the highest lipid peroxidation relative to HbO_2- and metHb-containing red cells, and that glucose inhibits both lipid peroxidation and haemoglobin damage (Trotta *et al.*, 1981). Treatment with NEM totally abolished glucose protection against lipid peroxidation but only partially blocked glucose protection of haemoglobin ($P < 0.01$ for metHb formation, $P < 0.05$ for non-intact Hb formation; five paired experiments). Ascorbate-enrichment further decreased lipid peroxidation in the presence of glucose ($P < 0.05$; four paired experiments) and this effect was partly abolished by treatment with NEM. Extracellular ascorbate caused at least 35% inhibition of lipid peroxidation regardless of the presence or absence of glucose or pretreatment with NEM. When both glucose and glutathione were available, a synergistic effect between glucose and extracellular ascorbate resulted in 86% inhibition of lipid peroxidation. In HbCO-containing red cells (as in HbO_2- and metHb-containing red cells) neither ascorbate-enrichment nor extracellular ascorbate had any effect on haemoglobin changes.

HbO_2- and HbCO-containing red cells are similar

Table 4. *Effects of t-butyl hydroperoxide on HbCO-containing red cells*

A 1% (v/v) suspension of red cells containing HbCO was exposed to 0.1 mM-t-butyl hydroperoxide for 1 h at 37°C. Conditions and procedures are described in the legend to Table 2.

		TBAR products (nM)*		Haemoglobin† MetHb (%)		Haemoglobin† Non-intact Hb (%)		Flux through the HMS (μmol of CO_2/h per ml of red cells)‡	Residual t-BuOOH (mM)§
Red cell preparations	Additions to incubation medium	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2		
Control	None	567 ± 106	623	28.3 ± 1.2	31.0	23.8 ± 2.1	17.6		0.013
Control	Ascorbate		345		32.2		15.1		0.014
Ascorbate-enriched	None	580 ± 96		28.9 ± 1.8		22.8 ± 1.5			0.014
NEM-treated	None	597 ± 112	689	29.8 ± 1.5	32.3	22.6 ± 1.5	19.5		0.016
NEM-treated	Ascorbate		437		31.0		18.6		0.016
Ascorbate-enriched/ NEM-treated	None	567 ± 104		29.0 ± 2.2		26.6 ± 0.7			0.016
Control	Glucose	398 ± 100	542	8.7 ± 1.6	7.3	5.2 ± 2.7	0.1	2.75	0.010
Control	Glucose + ascorbate		89		6.1		1.3	2.90	0.013
Ascorbate-enriched	Glucose	330 ± 95		7.9 ± 1.3		6.1 ± 1.1		2.58	0.010
NEM-treated	Glucose	550 ± 147	820	19.3 ± 1.4	26.4	14.8 ± 3.3	11.9	0.37	0.037
NEM-treated	Glucose + ascorbate		514		23.9		12.8	0.33	0.026
Ascorbate-enriched/ NEM-treated	Glucose	520 ± 133		21.4 ± 2.6		18.9 ± 1.5		0.40	0.029

* Data are means of four experiments ± S.E.M. (set 1) or two experiments (set 2).

† Data are means of five experiments ± S.E.M. (set 1) or one experiment (set 2).

‡ Data are means of three experiments. Flux through the HMS in the absence of t-butyl hydroperoxide was 0.32 ± 0.05 (mean ± S.E.M., $n = 9$) in control preparations and 0.18 ± 0.01 ($n = 9$) in NEM-treated preparations.

§ Data are means of two experiments.

in the response of the hexose monophosphate shunt to incubation with t-butyl hydroperoxide. In HbCO-containing red cells, t-butyl hydroperoxide caused an 8-fold increase in flux through the hexose monophosphate shunt. HbCO-containing red cells exhibited no inhibition of t-butyl hydroperoxide-induced stimulation of shunt activity by ascorbate-enrichment or extracellular ascorbate (unlike HbO_2-containing red cells), whereas pretreatment with NEM inhibited most of the shunt stimulation. There was significant residual t-butyl hydroperoxide after 1 h at 37°C in incubations with HbCO-containing red cells. Red-cell suspensions incubated in the presence of glucose had the lowest residual t-butyl hydroperoxide (10%), whereas suspensions of red cells pretreated with NEM and incubated in the presence of glucose had the highest (37%).

Stoichiometry of oxidative reactions in red-cell suspensions exposed to t-butyl hydroperoxide

Table 5 shows the total flux (in μmol) of oxidative reactions occurring in 1 h at 37°C in a reaction flask containing 2 ml of 1% (v/v) red-cell suspension. Table 5 allows direct comparisons between sources of reducing equivalents (electrons), namely, the intracellular glutathione pool, flux through the hexose monophosphate shunt and haemoglobin oxidation, and the need for reducing equivalents to reduce and thereby detoxify t-butyl hydroperoxide and its products. The formation of metHb from either HbO_2 or HbCO and non-intact Hb from metHb are each assumed to be one-electron oxidations. The reduction of t-butyl hydroperoxide (directly or indirectly to its formal products t-butyl alcohol and water) is assumed to be a two-electron reduction. By such calculations, consumption of t-butyl hydroperoxide can be completely accounted for in suspensions of HbO_2- and HbCO-containing red cells. MetHb-containing red cells show evidence of other routes of t-butyl hydroperoxide consumption. Comparison with levels of lipid peroxidation in Tables 2, 3 and 4 shows that lipid peroxidation is more dependent on the route of reduction of t-butyl hydroperoxide and its products than on the rate or completion of such reduction by 1 h.

Interaction of t-butyl hydroperoxide and ascorbate in non-cellular experiments

We observed a direct interaction between ascorbate and t-butyl hydroperoxide (Fig. 1). In incubations of ascorbate with t-butyl hydroperoxide but excluding red cells, ascorbate was oxidized at a rate three times faster than in the absence of t-butyl hydroperoxide. No reduced ascorbate was present after a 12 min incubation at 37°C. In addition, there was a loss of total ascorbate (defined as reduced ascorbate plus dehydroascorbate plus dioxogulonate) when ascorbate was incubated with t-butyl hydro-

Table 5. *Stoichiometry of oxidative reactions in red cell suspensions exposed to t-butyl hydroperoxide*
The total flux (in μmoles) of oxidative reactions occurring in 1 h at 37°C in a reaction flask containing 2 ml of 1% (v/v) red cell suspension is shown. At zero time each reaction vial contained 0.2 μmol of t-butyl hydroperoxide and 0.412 μmol of haem. The formation of non-intact Hb is assumed to occur through two one-electron oxidations: $Hb(Fe^{2+})(HbO_2$ or HbCO)→metHb and metHb→non-intact Hb. For red cells containing 100% metHb after treatment with NO_2^-, red cell reductase activity caused conversion of metHb into a maximum of 25% HbO_2 after pre-incubations and a final incubation of 1 h at 37°C in the absence of t-butyl hydroperoxide; incubation in the presence of t-butyl hydroperoxide caused conversion of all this HbO_2 into metHb as shown in the column labelled '$Hb(Fe^{2+})$→metHb'. The cellular GSH pool is the reduced glutathione present at zero time. By 1 h at 37°C in the presence of t-butyl hydroperoxide most of the glutathione was present in the oxidized form. Flux through the hexose monophosphate shunt (HMS) is expressed as μmol of electrons where 4 μmol of electrons is equivalent to 1 μmol of CO_2 produced. The reduction of t-Bu-OOH (directly or indirectly to its formal products t-butyl alcohol and water) is assumed to be a two-electron process. The theoretical capacity for haemoglobin and products of the hexose monophosphate shunt to reduce t-butyl hydroperoxide is calculated as follows: metabolized t-Bu-OOH (theoretical) = [($Hb(Fe^{2+})$→metHb) + (metHb→non-intact Hb) + (cellular GSH pool) + (flux through the HMS)]/2.

			Oxidation of haemoglobin				Theoretical		
Red cell haemoglobin	Red cell preparation	Additions to incubation medium	$Hb(Fe^{2+})$→metHb (μmol of haem)	metHb→non-intact Hb (μmol of haem)	Cellular GSH pool (μmol)	Flux through the HMS (μmol of electrons)	Metabolized t-Bu-OOH (μmol)	Residual t-Bu-OOH (μmol)	Actual residual t-Bu-OOH (μmol)
HbO_2	Control	None	0.325	0.087	0.045	None	0.229	0	<0.010
HbO_2	NEM-treated	None	0.338	0.095	None	None	0.217	0	<0.010
HbO_2	Control	Glucose	0.181	0.045	0.045	0.240	0.256	0	<0.010
HbO_2	NEM-treated	Glucose	0.325	0.099	None	0.037	0.231	0	<0.010
metHb	Control	None	0.078	0.168	0.045	None	0.146	0.054	<0.010
metHb	NEM-treated	None	0.054	0.188	None	None	0.121	0.079	0.028
metHb	Control	Glucose	0.095	0.148	0.045	0.062	0.175	0.025	<0.010
metHb	NEM-treated	Glucose	0.078	0.179	None	0.038	0.148	0.052	<0.010
HbCO	Control	None	0.215	0.098	0.045	None	0.179	0.021	0.026
HbCO	NEM-treated	None	0.216	0.093	None	None	0.155	0.045	0.032
HbCO	Control	Glucose	0.057	0.021	0.045	0.220	0.172	0.028	0.020
HbCO	NEM-treated	Glucose	0.140	0.061	None	0.030	0.116	0.084	0.074

peroxide. These reactions were presumably catalysed by trace metals because they were completely inhibited by DETAPAC. In experiments with red cells, inhibition of lipid peroxidation by ascorbate cannot be completely accounted for by simple inactivation of t-butyl hydroperoxide because only about 8% of t-butyl hydroperoxide is consumed in its reaction(s) with ascorbate and its products. In addition, ascorbate inhibition of lipid peroxidation occurred independently of haemoglobin changes; haemoglobin changes would be inhibited in parallel with inhibition of lipid peroxidation by ascorbate if protection was by scavenging of t-butyl hydroperoxide directly.

Discussion

In our previous study of t-butyl hydroperoxide-induced lipid peroxidation in red cells we demonstrated that lipid peroxidation and haemoglobin degradation represented the two extremes of a spectrum of oxidative damage (Trotta *et al.*, 1981). MetHb-containing red cells exhibited high levels of haemoglobin degradation and a relative sparing of membrane lipids. Glucose metabolism actually increased lipid peroxidation in HbO_2-containing red cells, apparently by preventing accumulation of the highly protective metHb. The underlying protective role of glucose metabolism against lipid peroxidation was demonstrated in HbCO-containing red cells, where the reactivity of the haem group was relatively diminished. In the present study, measurements of the flux of glucose through the hexose monophosphate shunt and consumption of t-butyl hydroperoxide have confirmed and amplified these findings. In suspensions of HbCO-containing red cells, in which haem is relatively unavailable, t-butyl hydroperoxide was not completely consumed by 1 h at 37°C. In the presence of glucose, hexose monophosphate shunt activity replaced haem reactivity as the main route of t-butyl hydroperoxide consumption but did not increase net consumption. The results were similar with HbO_2-containing red cells, except that the greater availability of haem resulted in complete consumption of t-butyl hydroperoxide. In metHb-containing red cells, the con-

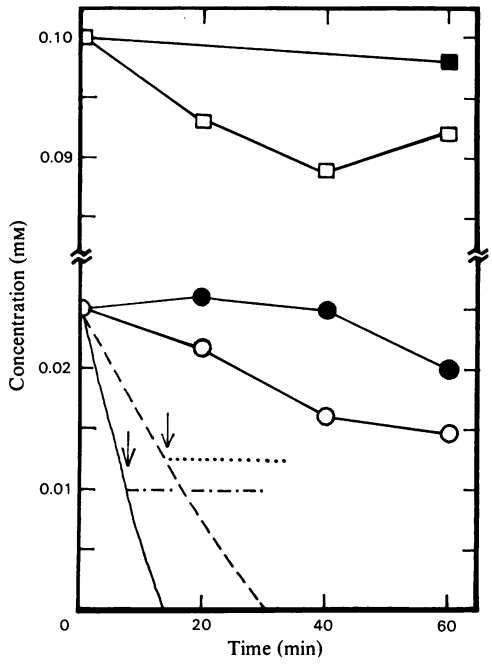

Fig. 1. *Reaction of ascorbate with t-butyl hydroperoxide*
Ascorbate (0.025mM) was incubated with or without t-butyl hydroxyperoxide (0.1mM) in Krebs–Ringer phosphate buffer (pH 7.4) at 37°C. In some incubations, DETAPAC (0.2mM) was added either at zero time (filled symbols) or at a later time as indicated by an arrow in the Figure. Samples were taken at 0, 20, 40 and 60min for measurement of total ascorbate (defined as reduced ascorbate plus dehydroascorbate plus dioxogulonate) and t-butyl hydroperoxide as described in the Materials and methods section. Levels of reduced ascorbate were monitored continuously by measuring A_{266} in a Cary 14 spectrophotometer. Levels of t-butyl hydroperoxide were measured in the presence of ascorbate (□) or ascorbate plus DETAPAC (■). Levels of total ascorbate were measured in the presence of t-butyl hydroperoxide (○) or t-butyl hydroperoxide plus DETAPAC (●). Levels of reduced ascorbate: ---- ascorbate alone; ···· ascorbate plus DETAPAC; ——, ascorbate plus t-butyl hydroperoxide; -·-·-· ascorbate plus t-butyl hydroperoxide plus DETAPAC.

version of metHb into non-intact Hb accounted for most of the consumption of t-butyl hydroperoxide with little role for hexose monophosphate-shunt activity. The conversion of metHb into non-intact Hb appears to account for more t-butyl hydroperoxide consumption than a simple one-electron transfer would suggest. Further oxidation of non-intact Hb may involve protein precipitation and oxidation of sulphydryl groups and result in binding of haemoglobin to the membrane (Peisach *et al.*, 1975; Trotta *et al.*, 1981). Oxidation of haemoglobin sulphydryl groups by the following reaction with GSSG would result in regeneration of reduced glutathione and hence reducing equivalents for the enzymic metabolism of t-butyl hydroperoxide:

$$GSSG + Hb\text{–}SH \rightarrow Hb\text{–}SS\text{–}G + GSH$$

Although consumption of t-butyl hydroperoxide by the hexose monophosphate shunt protected against lipid peroxidation, the final extent of lipid peroxidation was more dependent on the balance between the presence of haem catalysts (HbO_2 and low concentrations of metHb) and inhibitors (high concentrations of metHb) of lipid peroxidation (Trotta *et al.*, 1981). In support of these findings, it has been demonstrated that hydroperoxides are stable and unable to initiate chains of peroxidation in lipids in the absence of catalysts such as Fe^{2+} or haem iron (see Mead, 1976). Several haem proteins have been shown to catalyse decomposition of hydroperoxides (O'Brien, 1969) and inhibit lipid peroxidation (Lewis & Wills, 1963). Metmyoglobin has been shown to reduce cumene hydroperoxide to form the oxy-radical and to reduce the oxy-radical to form the alcohol (Griffin & Ramirez, 1981). Our results indicate that metHb is able to effect reduction of t-butyl hydroperoxide, efficiently scavenge any free-radical products of this reduction and spare membrane lipids from peroxidation. These reactions probably result in higher oxidation states of haem (see George, 1953) and subsequent formation of non-intact Hb forms, probably haemichromes (Peisach *et al.*, 1975; Trotta *et al.*, 1981). In HbO_2- and HbCO-containing red cells, the one-electron oxidation of ferro- to ferri-haemoglobin (metHb) by t-butyl hydroperoxide implies heterolytic scission of the O–O bond to form t-butyloxy radicals. t-Butyloxy radicals are capable of abstracting allylic hydrogens from unsaturated fatty acid residues of phospholipids and initiating lipid peroxidation. In addition, t-butyloxy radicals can be rapidly reduced by ascorbate (Bors *et al.*, 1981) or may be scavenged by accumulated metHb.

Glutathione plays a key role in scavenging t-butyl hydroperoxide and mediating the protective effect of the hexose monophosphate shunt against lipid peroxidation and haemoglobin degradation. The primary mechanism by which glutathione metabolizes t-butyl hydroperoxide is probably through the activity of glutathione peroxidase, which has been shown to accept t-butyl hydroperoxide as a substrate (Srivastava *et al.*, 1974). Glutathione peroxidase-dependent stimulation of hexose monophosphate-shunt activity by alkyl hydroperoxides has been demonstrated previously in hepatocytes (Sies & Moss, 1978), haemoglobin-free perfused liver (Sies & Summer, 1975) and adipocytes (May, 1981); this stimulation reflects changes in glu-

tathione redox state and the resulting oxidation of NADPH (Oshino & Chance, 1977). In the presence of glucose, flux through the hexose monophosphate shunt accounted for a maximum consumption of t-butyl hydroperoxide (percentage of total) of 60% in HbO_2-containing red cells, 55% in HbCO-containing red cells and 16% in metHb-containing red cells. Inactivation of glutathione by treatment with NEM decreased the maximum possible hydroperoxide consumption by the hexose monophosphate shunt to less than 10% in all three red-cell types. In HbCO-containing red cells, inactivation of glutathione completely inhibited glucose protection against lipid peroxidation, but only partially inhibited glucose protection of haemoglobin. In HbO_2-containing red cells, inactivation of glutathione increased the level of lipid peroxidation in the presence of glucose (uncovering the underlying protective effect of the hexose monophosphate shunt) and completely inhibited glucose protection of haemoglobin. The partial protection of haemoglobin by glucose in HbCO-containing red cells in the absence of glutathione may represent the effect of metHb reductase activity, which is dependent on glycolysis and leads to conversion of metHb into deoxyhaemoglobin and then into the relatively inert HbCO.

Neither ascorbate-enrichment nor extracellular ascorbate had any effect on either consumption of t-butyl hydroperoxide or oxidation of haemoglobin. Ascorbate seems to be mainly involved in scavenging chain-propagating species involved in lipid peroxidation. Ascorbate far exceeds sulphydryl groups, phenolic groups (tocopherol) and several other antioxidants in its ability to scavenge alkoxy radicals (Bors *et al.*, 1981), a propagative intermediate of lipid peroxidation chain reactions. Intracellular ascorbate inhibited lipid peroxidation but required glucose metabolism to maintain ascorbate in reduced form. Ascorbate reduction by glucose metabolism was approx. 50% glutathione-dependent. Extracellular ascorbate supplies a large stoichiometric source of reducing equivalents without requirement of glucose metabolism, although a synergistic inhibitory effect on lipid peroxidation was observed between ascorbate and glutathione in HbCO-containing red cells. There is evidence supporting a glutathione-dependent enzymically catalysed reduction of dehydroascorbate to ascorbate in red cells (Hughes, 1964). Ascorbate also decreased flux through the hexose monophosphate shunt in red cells containing HbO_2 but had no net effect on t-butyl hydroperoxide consumption. This last finding suggests that in HbO_2-containing red cells, ascorbate may scavenge reactive products of the reaction between t-butyl hydroperoxide and HbO_2.

t-Butyl hydroperoxide-induced lipid peroxidation in red cells is not dependent on the rate or completion of t-butyl hydroperoxide consumption but rather on the route of t-butyl hydroperoxide consumption. Lipid peroxidation appears to depend on the balance between the presence of initiators of lipid peroxidation ($e + t\text{-}Bu\text{-}OOH \rightarrow t\text{-}Bu\text{-}O + OH^-$) such as HbO_2 or low concentrations of metHb (Griffin & Ramirez, 1981; Trotta *et al.*, 1981) and terminators of lipid peroxidation such as glutathione, reduced ascorbate and high concentrations of metHb (Trotta *et al.*, 1981) which scavenge either t-butyloxy radicals ($e + t\text{-}Bu\text{-}O + H^+ \rightarrow t\text{-}Bu\text{-}OH$) (Bors *et al.*, 1981) or other intermediates of propagative chains sustaining lipid peroxidation (Griffin & Ramirez, 1981).

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